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(21) International Application Number: PCT/US97/12181 (22) International Filing Date: 15 July 1997 (15.07.97) (30) Priority Data: 60/022,622 26 July 1996 (26.07.96) US (71) Applicant: BRISTOL-MYERS SQUIBB COMPANY [US/US]; P.O. Box 4000, Princeton, NJ 08543-4000 (US). (72) Inventors: USHER, John, J.; 486 Summerhaven Drive, East Syracuse, NY 13057 (US). ROMANCIK, Guna; 6103 Thunderhead Lane, Jamesville, NY 13078 (US). (74) Agents: ALMAULA, Prabodh, I. et al.; Bristol-Myers Squibb Company, P.O. Box 4000, Princeton, NJ 08543-4000 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: SYNTHESIS OF β -LACTAM ANTIBACTERIALS USING SOLUBLE SIDE CHAIN ESTERS AND ENZYME ACYLASE (57) Abstract Disclosed is a process for the synthesis of β -lactam antibacterials using soluble side chain esters in the presence of enzyme acylase. Also disclosed are novel esters useful as reactants in said process.		

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5 SYNTHESIS OF β -LACTAM ANTIBACTERIALS USING SOLUBLE SIDE CHAIN ESTERS AND ENZYME ACYLASE

This application claims priority from provisional U.S. Application Serial No.
60/022,622, filed July 26, 1996, incorporated herein by reference in its entirety.

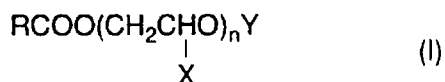
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Field of the Invention

The present invention is directed to a process for the production of antibacterial β -
lactams, namely penicillins and cephalosporins, in the presence of the enzyme, acylase, and a
15 soluble ester as the acyl source. The invention is also directed to novel esters useful as
reactants in said process.

Description of Related Art

20 Japanese Patent Application Publication Nos. 47-25388, 47-29588, 48-26985, 48-
35090, 48-99393, 49-14687, 49-36890, 49-48892, 49-75787, 49-134893 and 52-110896 teach
processes for preparing penicillins or cephalosporins by reacting a methyl or ethyl ester of the
acyl moiety to be introduced, with 6-aminopenicillanic acid or 7-aminocephalosporanic acid or
derivatives thereof. The acyl source in these references is a lower alkyl ester and especially
25 a methyl ester. Eiji Kondo, et al., U.S. Patent No. 4,340,672, issued July 20, 1982, discloses a
process for preparing antibacterial β -lactams, that is penicillins and cephalosporins, by the
action of an acylase on a penicillin or cephalosporin nucleus amine and an ester(1) of the
following formula as the acyl source



30 wherein RCO is an acyl group of penicillin or cephalosporin side chains; X is a hydrogen atom,
lower alkyl group or hydroxy lower alkyl group; Y is a hydrogen atom or a lower alkyl group;
and n is an integer from 1 to 20. The foregoing process uses the (poly)ethyleneglycol ester as
the acyl source which is freely miscible with water as compared to known acyl sources, such
as methyl esters, which have low solubility.

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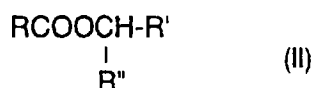
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- 5 WO 92/01061, assigned to Novo Nordisk and published January 23, 1992, relates to a process for the preparation of β -lactam derivatives by enzymatic acylation of the parent amino β -lactam with amides as acylating agents.

Summary of the Invention

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The present invention provides a novel process for preparing antibacterial β -lactams, that is penicillins and cephalosporins, by the action of an acylase on a penicillin or cephalosporin nucleus amine and an ester having the formula (II) as the acyl source



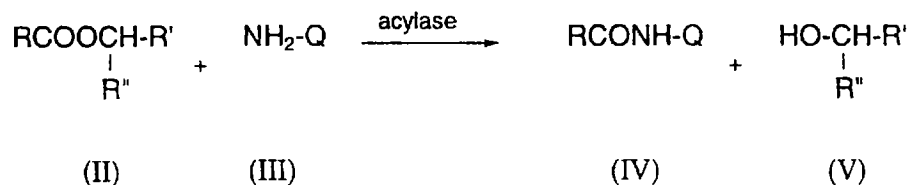
- 15 wherein RCO is a penicillin or cephalosporin side chain acyl group; R' is H or CH_2X or CHXY ; R'' is H or CH_2Y or CHXY and X and Y each are independently hydrogen, hydroxy, lower alkyl or hydroxy-lower alkyl.

Detailed Description of the Invention

20

As used in the specification, unless otherwise indicated explicitly or by context, the acyl group represented by RCO is an acyl of natural or synthetic, penicillins or cephalosporins. The lower alkyl includes alkyl of 1 to 4 carbon atoms.

- 25 One aspect of the present invention provides a process for the production of β -lactam antibacterials by reacting an ester of the formula (II) with aminoazetidinone carboxylic acid of the formula (III) in the presence of acylase in an aqueous medium to produce the β -lactam antibacterial of the formula (IV)



30

wherein RCO is an acyl group, $\text{NH}_2\text{-Q}$ is an amino source and acylase is an enzyme of bacterial or fungal origin or obtained by recombinant methods. The substituents R' and R'' are as defined above. The acyl group RCO can be straight, branched, cyclic, or partially

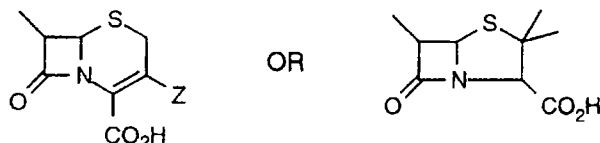
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- 5 cyclic lower alkanoyl or lower alkenoyl; monocyclic lower aralkanoyl, monocyclic
 aryloxylower alkanoyl, (O, N, or S)-heterocyclic-lower alkanoyl, (O, N, or S)-heterocyclic
 thio-lower alkanoyl, cyanoacetyl, cyanomethyl-thioacetyl, monocyclic arylglycyl, monocyclic
 cycloalkenylglycyl, monocyclicaryl glycolyl, N-acyl-aryl glycyl, monocyclicarylmalonyl or
 arylsulfoalkanoyl, all above optionally having lower alkyl, aminomethyl, halogen, hydroxy,
 10 lower alkanoyloxy or lower alkoxy as a substituent, and preferably containing 1 to 15 carbon
 atoms.

The preferred RCO groups are D-phenylglycyl and 4-hydroxy-D-phenylglycyl which
 are well known as side chains of antibiotics such as cephalexin and amoxicillin.

15

The amino source $\text{NH}_2\text{-Q}$ refers to the substituent on the 7-amino group or 6-amino
 group of known cephalosporin or penicillin antibiotics, Q having the formula (VI)



(VI)

- wherein Z is hydrogen, halogen, lower alkyl of 1-3 carbon atoms, haloalkyl of 1-3 carbon
 20 atoms, C_{2-4} alkenyl, a nucleophilic group or lower alkyl of 1-3 carbon atoms substituted by a
 nucleophilic group. The halogen atom is chlorine, bromine or iodine and the representative
 nucleophilic groups are disclosed in Japanese Patent Application Publication No. 49-81381.

- The enzyme acylase can be of plant, animal, fungal or bacterial origin or obtained by
 25 recombinant methods. The bacterial or fungal acylases are especially important as far as
 production, efficiency, cost and stability are concerned.

- Representative bacteria or fungi for the acylase source include strains of
 microorganisms belonging to, for example, genera Acetobacter, Achromobacter, Aeromonas,
 30 Alkaligenes, Arthrobacter, Brevibacterium, Beneckea, Bacillus, Corynebacterium,
Escherichia, Flavobacterium, Gluconobacter, Kluyvera, Microbacterium, Micrococcus,
Nocardia, Proteus, Pseudomonas, Rhodopseudomonas, Spirillum, Staphylococcus,
Xanthomonas, Aphanocladium or Cephalosporium, or natural or artificial mutants or variants

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5 of them capable of producing acylase for the reaction of this invention. Such strains include those described in, for example, *Advances in Applied Microbiology*, Volume 20, page 217 (1976), or natural or artificial mutants of them available for the reaction of this invention. An especially useful enzyme is penicillin G amidase which can be obtained from Escherichia coli by methods which are well known in the art. It can also be obtained by use of
 10 recombinant E. Coli containing the penicillin G amidase gene with a tac promoter by methods which are well known in the art. This method is preferred since it makes available large quantities of the enzyme suitable for the manufacturing process.

The aminoazetidinone carboxylic acid of formula (III) can be used as water soluble
 15 alkali metal salts, such as sodium, lithium and potassium, or as an ester, such as methanesulfonyl ethyl ester or acetyl ester. The aminoazetidinones of formula (III) and its salts or esters are known compounds and can be prepared by known methods.

The esters of formula (II) can be prepared by known methods, that is, by reaction of
 20 an acid RCOOH with a diol or triol or polyol of the formula (V)

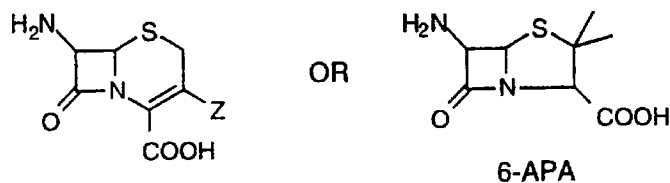


wherein RCO, R' and R'' are as defined above. The esterification reaction can be by dehydration under acid catalyzed conditions or by means of acid halide or by ester exchange reactions. The RCOOH and the diol, triol or polyol are known compounds or can be obtained
 25 by methods which are well known in the art.

Another aspect of the invention provides the ester R-COO-CH₂-CH₂-OH wherein R is D-Phenylglycyl or 4-hydroxy-D-phenylglycl. These esters are novel and important reactants in the process of the invention because they provide yields of over 90% of the final products.
 30 These esters are prepared by known methods.

A preferred embodiment of the present invention is a process for preparing antibacterial β -lactams, that is, cefprozil, cefadroxil and amoxicillin by the action of penicillin G amidase on penicillin or cephalosporin amines of the following formula (VII)

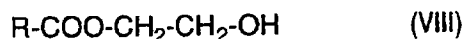
- 5 -



(VII)

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wherein Z is methyl (in 7-ADCA) or 1-propenyl (in 7-PACA, the cefprozil nucleus) group and an ester of the formula (VIII)



10 wherein R is 4-hydroxy-D-phenylglycyl. The penicillin or cephalosporin amines of the formula (VII) are known compounds and can be obtained by methods well known in the art. The ester of formula (VIII) can be obtained by reacting RCOOH wherein R is defined as above with ethylene glycol. The compound RCOOH and ethylene glycol are commercially available.

15 The process of the invention is carried out by preparing a solution containing ester, cephalosporin or penicillin amine and the enzyme without a buffer at room temperature. This solution is prepared by dissolving the ester in water and adding ammonium hydroxide until a pH of 7.5 is reached. Then the penicillin or cephalosporin amine is added to the ester solution and the pH again adjusted to 7.5 with ammonium hydroxide. The mixture is then cooled to 5-15°C and the solid enzyme is added to it. During this time, the pH falls about 0.6 units and is

20 not maintained at 7.5. The reaction mixture is then analyzed by high pressure liquid chromatography, for example on a C18 reversed phase column, 5 cm X 4.6 mm, 5µm spherosorb ODS2. The final product is obtained in a yield of 90-99%.

25 The process of this invention gives the final antibacterial β-lactam in the usual yield of over 90% and sometimes over 95% and occasionally over 99%. The yield obtained by simple alkyl esters, such as methyl are usually in the range of 70 to 90%. Because of the high yield of the final antibiotic obtained by the present process, the process is very useful for the manufacture of penicillin or cephalosporin antibiotics.

30 A typical reaction mixture contains 5-35% of the acyl source (VIII) (preferably 8-12%) and 2-20% of the amine source (VII) (preferably 2-5%). The enzyme can be present in a soluble or insoluble form, but preferably the enzyme is present in the form of an insoluble immobilized preparation. This preparation has advantages, such as improved stability of the

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- 5 enzyme and also relative ease of removal of immobilized enzyme from reaction mixtures as a first step in the isolation of the antibiotic.

The invention is illustrated but in no way limited by the following Examples:

10

Example 1

Synthesis of cefprozil from (VII) using immobilized recombinant penicillin G amidase as the enzyme and hydroxyethyl ester of 4-hydroxy-D-phenylglycine prepared by acid catalyzed reaction as the acyl source

15

A mixture of 4-hydroxy-D-phenylglycine (10 g), ethylene glycol (15 ml) and concentrated sulphuric acid (5 ml) was stirred for 18 hours at 55°C under anhydrous conditions. The solution was cooled, and then ice (10 g) was added to it, and the pH was adjusted to 1.0 with 10 N-NH₄ OH (4.5 ml) giving 40 ml of solution of hydroxyethyl ester.

20

The enzyme mixture of 20 ml containing 10% ester, 4% (VII), and 8% enzyme (equivalent to 32 IU/ml of enzyme) was made up without buffer as follows:

25

The above prepared ester solution (6.9 ml) was mixed with water (2 ml) and adjusted to pH 7.5 with 10 N-NH₄OH. Then the compound (VII) (0.8 g) was added to it and the pH adjusted to 7.5 with 1 N-NH₄OH and the volume to 18.4 ml. Then the mixture was cooled to 5-15°C and solid enzyme (1.6 g; 640 IU) was added to it. The pH was not maintained at 7.5 and fell about 0.6 units during the reaction. The reaction mixture was analyzed by HPLC on a C18 Reverse Phase column 5 cm X 4.6 mm, 5µm spherosorb ODS2. The mobile phase was 10% acetonitrile/0.3% H₃PO₄ @ 2 ml/minute with 215 nm detection. The isomers of cefprozil appeared at 2.9 minutes (cis) and at 5.1 minutes (trans). The final product was obtained with a maximum yield of 92-96%. The whole experiment was completed in 25-50 minutes.

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Example 2

Synthesis of cefprozil from (VII) using immobilized recombinant penicillin G amidase as the enzyme and purified hydroxyethyl ester of 4-hydroxy-D-phenylglycine as the acyl source

The purified ester was prepared by the reaction of the Dane salt of 4-hydroxy-D-phenylglycine with bromoethanol and was obtained as a pure crystalline hydrochloride salt.

The reaction mixture of 20 ml containing 12.5% ester, 4% (VII), and 8% enzyme (equivalent to 24 IU/ml of enzyme) was prepared as follows:

The purified ester (2.5 g), prepared as above, was mixed with water (9 ml) and adjusted to pH 8.0 with concentrated NH_4OH to give a solution. The compound (VII) (0.8 g) was added to the solution and the pH of the solution adjusted to 6.76 with 2M- H_2SO_4 and the volume to 18.4 ml. This solution was cooled to 15°C and then solid enzyme (1.6 g; 480 IU) was added to it. The pH was not maintained and fell about 0.6 units during the reaction. The final product was obtained in a maximum yield of 99%. The area percent of unreacted (VII) fell to 0.7%. The experiment was completed in twenty-five minutes.

15

Example 3

Synthesis of cefadroxil from 7-ADCA using Boehringer penicillin G amidase as the enzyme and hydroxyethyl ester of 4-hydroxy-D-phenylglycine prepared by acid catalyzed reaction as the acyl source

The ester was prepared as in Example 1. The experiment was repeated as in Example 1 but at 15°C and using 1.6 g of the Boehringer enzyme. The reaction mixture was analyzed by HPLC on a Merck column, 250-4, LiChrosorb RP-18 (10 μm), with 10% acetonitrile/1% $\text{NH}_4\text{H}_2\text{PO}_4$ as mobile phase run at 1.3 ml/minute with 280nm detection. The product was obtained with a maximum yield of ~95%. The experiment was completed in 35 minutes.

30

Example 4

Synthesis of amoxicillin from 6-APA using Boehringer penicillin G amidase as the enzyme and hydroxyethyl ester of 4-hydroxy-D-phenylglycine prepared by acid catalyzed reaction as the acyl source

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A mixture of volume 20 ml containing 12% ester, 3% 6-APA, and 8% enzyme was prepared as follows:

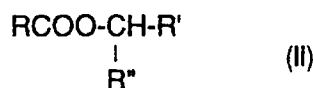
10 A solution of the ester (prepared as in Example 1, 8.3 ml) was mixed with water (2 ml) and adjusted to pH 7.5 with 10 N-NH₄OH. Then the compound 6-APA (0.6 g) was added to the solution and the pH adjusted to ~7.0 with 1N ammonium hydroxide to a volume to 18.4 ml. Then the solution was cooled to 20°C and Boehringer enzyme (1.6 g) was added to it. This solution was analyzed by HPLC on a C18 Reverse Phase column 5cm X 4.6 mm, 5 µm spherosorb ODS 2. The mobile phase was 2.5% acetonitrile/0.025M NaH₂PO₄ to pH 3.5 with H₃PO₄ run @ 1 ml/minute with 215 nm
15 detection. The final product was obtained in a yield of 90-95% in 160 minutes.

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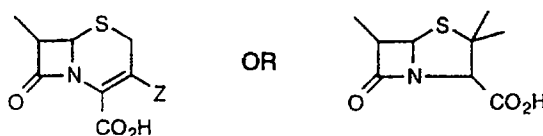
Claims

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1. A process for producing a penicillin or cephalosporin comprising reacting an amine of the formula $\text{NH}_2\text{-Q}$ (III), with an ester of the formula (II)



10 in the presence of an acylase,
wherein Q is



wherein Z is hydrogen, halogen, lower alkyl of 1-3 carbon atoms, haloalkyl of 1-3 carbon atoms, C_{2-4} alkenyl, a nucleophilic group or lower alkyl of 1-3 carbon atoms substituted by a nucleophilic group, RCO is straight, branched, cyclic, or partially cyclic lower alkanoyl or lower alkenoyl, monocyclic lower aralkanoyl, monocyclic aryloxylower alkanoyl, (O, N, or S)-heterocyclic-lower alkanoyl, (O, N, or S)-heterocyclic thio-lower alkanoyl, cyanoacetyl, cyanomethyl-thioacetyl, monocyclic arylglycyl, monocyclic cycloalkenylglycyl, monocyclic arylglycolyl, N-acyl-arylgylyl, monocyclic arylmalonyl or arylsulfoalkanoyl, all above optionally having lower alkyl, aminomethyl, halogen, hydroxy, lower alkanoyloxy or lower alkoxy as a substituent, and containing 1 to 15 carbon atoms. R' is hydrogen, CH_2X or CHXY , R'' is hydrogen, CH_2Y or CHXY , wherein X and Y each are independently hydrogen, hydroxy, lower alkyl or hydroxy-loweralkyl, to produce a penicillin or cephalosporin of the formula (IV)

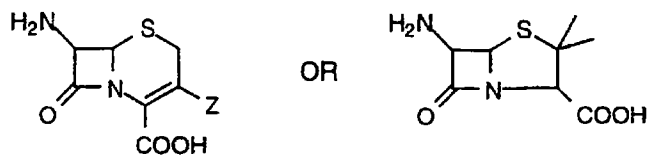
25 RCONH-Q (IV), wherein RCO and Q are as defined above.

2. A process of claim 1, wherein $\text{NH}_2\text{-Q}$ is a water soluble alkali metal salt or an ester.

30 3. A process of claim 1, wherein the acylase is immobilized recombinant penicillin G amidase.

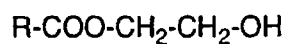
4. A process for producing antibiotic cefprozil, cefadroxil or amoxicillin, comprising reacting

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wherein Z is methyl or 1-propenyl group with an ester of the formula



wherein R is 4-hydroxy-D-phenylglycyl in the presence of the enzyme, penicillin G amidase to produce cefprozil, cefadroxil or amoxicillin.

10

5. The process of claim 4, wherein the enzyme is immobilized recombinant penicillin G amidase.

6. A compound of the formula $R-COO-CH_2-CH_2-OH$ wherein R is D-phenylglycyl or 4-hydroxy-D-phenylglycyl.

15

INTERNATIONAL SEARCH REPORT

 International application No.
PCT/US97/12181

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12P 37/00, 37/02, 35/00; C12N 15/00

US CL :435/43, 46, 47, 244, 230, 172.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/43, 46, 47, 244, 230, 172.3

 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 ENZYME NOMENCLATURE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FERNANDEZ-LAFUENTE et al., Synthesis of antibiotics (cephaloglycin) catalyzed by penicillin G acylase: Evaluation and optimization of different synthetic approaches Enzyme and Microbial Technology. 01 July 1996. Vol. 19, pages 9-14, especially page 11.	1-6
Y	CH 640240 (I.B.S.A. INSTITUT BIOCHIMIQUE S.A.) 30 December 1983, Example 9.	1-6
Y	WO 93/23164 A1 (NOVO NORDISK A/S) 25 November 1993), Example 2.	1-6
Y	WO 92/01061 A1 (NOVO NORDISK A/S) 23 January 1992, pages 6-8 and Examples.	1-6

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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Date of the actual completion of the international search

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Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT**International application No.**
PCT/US97/12181**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 93/12250 A1 (NOVO NORDISK A/S) 24 June 1993, Examples.	1-6
Y	WO 96/02663 A1 (GISTBROCADES B.V.) 01 February 1996, pages 7-9 and Examples.	1-6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/12181

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, DIALOG, BIOSIS

Search terms: penicillin?, cephalosporin?, acylase?, amine?, ester?, immobiliz?, immobilis?, recombinant, penicillin G
amidase, cefprozil, cefadroxil, amoxicillin

Structure search